EXHIBIT HH

Original article

Influence of implantation interval on the long-term biocompatibility of surgical mesh

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Background: The aim was to study the long term tissue response to polypropylene mesh.
Methods: This was a retrieval study that investigated 76 polypropylene meshes with a median implantation interval of 18 (range 2 180) months. Mesh was explanted following hernia recurrence, infection or pain. The median implantation interval was 20 (range 4 180) months in the recurrence group, 30 (range 5 48) months in the pain group and 10 (range 2 56) months in the infection group ($P < 0{\cdot}05$, infection *versus* pain or recurrence). The inflammatory response was determined by immunohistochemistry of macrophages (CD68), polymorphonuclear granulocytes (CD15) and T and B lymphocytes (CD3 and CD20). The cell turnover within the interface mesh fibre recipient tissue was measured by TUNEL for apoptosis or DNA strand breaks, Ki67 for cell proliferation and heat shock protein (HSP) 70 for cell stress.
Results: With the exception of HSP 70, levels of all variables decreased over time. Sex, age, type of previous operation or location of the mesh did not have a significant influence.
Conclusion: Long term incorporated polypropylene mesh in humans has a more favourable tissue response with increasing implantation interval.

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Introduction

In the 1960s the introduction of surgical mesh was a revolutionary and unorthodox step in hernia repair. Modern hernia surgery is no longer imaginable without the application of these biomaterials, leading to about one million implantations each year worldwide. The net like alloplastic mesh is used to close the hernia defect and, with extended overlap, to reinforce the abdominal wall.

Surgical mesh is regarded as inert and biocompatible. However, knowledge about the long term biocompatibility and tissue response of mesh in humans is still poor, although a few reports exist[1,2]. Nearly all the available data on the biological behaviour of these implants are from animal experiments[3–15].

The aim of the present study was to evaluate the biocompatibility of surgical mesh that was explanted as a result of a clinical complication such as infection, hernia recurrence or chronic pain. All mesh specimens were collected between 1995 and 2001 at different European centres of hernia surgery and sent to the Centre of Biomaterial and Implant Pathology, Aachen. The aim was to study the local tissue reaction at the interface between surgical mesh and recipient tissue, and to identify changes that occurred since implantation, similar to a previous study[16].

Materials and methods

Tissue samples

Seventy six full explants of a standard polypropylene mesh (Marlex®, CR Bard, Murray Hill, New Jersey, USA) were collected after revision operations at various hospitals in Europe. All mesh specimens were originally implanted for abdominal wall hernia repair. The mesh had been implanted in the groin (n = 49, 64 per cent) or in the anterior abdominal wall (n = 27, 36 per cent), either in the inguinal subfascial area using the Lichtenstein method (n = 29, 38 per cent), in the preperitoneal submuscular

space by open or laparoscopic procedures (n = 32, 42 per cent) or in front of the fascia in an onlay position (n = 15, 20 per cent). Immediately after explantation all mesh samples were fixed in 10 per cent buffered formalin at the donating centre and sent by mail to the Institute of Pathology in Aachen, together with relevant clinical data.

Morphological study

All mesh specimens were studied by light microscopy. Tissue samples were sliced into 0·3 × 1·0 cm pieces from representative lateral parts and from the centre of the mesh, and embedded in paraffin. Additionally, visibly striking areas, such as folds or node formations, were investigated. Between ten and 15 sections 4 μm thick were stained with haematoxylin and eosin, as well as with periodic acid Schiff plus diastase and Elastic van Gieson.

Immunohistochemistry

Light microscopy was complemented by immunohisto chemistry, which was performed on paraffin embedded material using the avidin biotin complex method with diaminobenzidine as chromogen. The procedure was repeated twice for each sample at different timepoints.

Antibodies

Antibodies used in this study included polyclonal rabbit antihuman CD3 (DAKO, Hamburg, Germany) 1 : 50 as pan marker for T lymphocytes, polyclonal rabbit antihu man CD20 (DAKO) 1 : 50 as pan marker for B lympho cytes, monoclonal mouse antihuman CD68 (DAKO) 1 : 50 as a pan marker for macrophages, monoclonal antihuman CD15 (Becton Dickinson, Heidelberg, Germany) 1 : 10 as a marker for polymorphonuclear granulocytes (PMNs), polyclonal rabbit anti heat shock protein (HSP) 70 (DAKO) 1 : 200 and monoclonal anti HSP 70/heat shock core protein 70 (BIOMOL, Hamburg, Germany) 1 : 200 as markers for the cell stress response, and monoclonal human Ki67 (= MIB1) (DIANOVA, Hamburg, Germany) 1 : 10 as a marker of cell proliferation.

TUNEL

TUNEL histochemistry was performed by means of an *in situ* apoptosis detection kit (Apoptag®; Oncor, Hamburg, Germany). All steps were done according to the supplier's instructions. Briefly, paraffin embedded sections 4 μm thick were taken from each mesh specimen, fixed to slides by heating at 60°C, deparaffinized and rehydrated. After digestion with 0·02 per cent trypsin in phosphate buffered saline (PBS) at room temperature and washing in PBS, the sections were incubated in buffer A (potassium cacodylate 200 mmol/l, Tris 0·025 mmol/l, bovine serum albumin 0·25 mg/ml, pH 6·6) for 5 min. The sections were then incubated with a labelling solution containing terminal digoxin labelled thymidine, biotinylated 16 2′ deoxyuri dine 5′ triphosphate and cobalt chloride 1·5 mmol/l in buffer A at 37°C for 1 h. The reactions were terminated by rinsing in a buffer containing sodium chloride 300 mmol/l and sodium citrate 30 mmol/l, pH 7. The sections were washed three times in PBS for 5 min. For light microscopy, the labelled DNA fragments were visualized by incubating the sections with streptavidin conjugated alkaline phos phatase followed by reaction with medium containing fast red as chromogen. The slides were then washed, counter stained with methyl green and mounted in Permount® medium (Merck, Darmstadt, Germany).

Morphometry

Morphometric evaluation consisted of a quantitative cell analysis of the inflammatory reaction and soft tissue reaction. Variables measured were the percentage share area of the inflammatory infiltrate (partial volume), connective tissue and vessels, as well as the percentage of macrophages, lymphocytes, PMNs and fibroblasts, includ ing the expression of TUNEL, Ki67 and HSP 70 in these cells.

The share areas were determined in five haematoxylin and eosin stained slides at a grid of ten fields (0 300 μm; 100 ×, area 0·1 mm^2) in ten random fields per slide. The number of cells was counted again with a grid of ten points in the interface mesh fibre recipient tissues (0 150 μm, 400 ×, area 625 μm^2) in ten fields per slide.

Statistical analysis

The influence of the clinical variables on the tissue response was tested for significance by analysis of variance with least significant difference modification according to Bonferroni. The Pearson correlation coefficient was calculated as an indicator of the association between the implantation interval and morphometric data. Multivariate analysis was used to verify independent effects for indica tion, implantation time, implantation technique and mesh location, patient sex and age. Statistical significance was assumed at $P < 0·05$. All data are shown as mean(s.d.) unless otherwise stated.

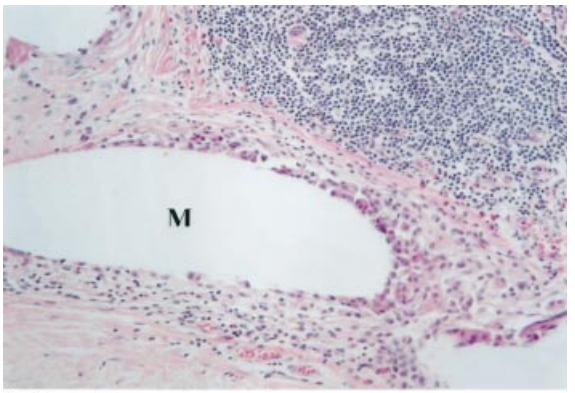


Fig. 1 Typical histological aspect after **a** 12 months and **b** 180 months of implantation; note the decreased inflammatory reaction at the interface and the surrounding scar formation after 180 months (**b**). M, mesh fibre (haematoxylin and eosin stain, original magnification × 200)

Results

Between 1995 and 2001 a total of 76 explanted specimens of polypropylene mesh (Marlex®) were collected. The mesh came from 52 men and 24 women with a mean age of 55(11) and 52(15) years respectively. Eight patients were below the age of 40 years. The main indications for explantation were infection (n = 19, 25 per cent), chronic pain (n = 10, 13 per cent) or hernia recurrence (n = 47, 62 per cent). The median duration of incorporation was 20 (range 4 180) months in patients who had mesh removal for recurrence, 30 (range 5 48) months for pain, and 10 (range 2 56) months for infection ($P < 0{\cdot}05$, infection *versus* pain or recurrence). There were no significant differences between the three groups with regard to implantation procedure or patient sex. Patients with an onlay mesh were significantly older (61(13) years) than those with a preperitoneal mesh (51(12) years) ($P < 0{\cdot}05$) or an inguinal, subfascial mesh (54(13) years). Some 28 per cent of all reoperations for recurrent hernia were performed within 1 year of mesh implantation, 58 per cent within the first 2 years and 24 per cent after 3 years. The rate of infection was similarly distributed between both sexes and all locations.

General macroscopic and microscopic observations (*Fig. 1* and *Table 1*)

All removed polypropylene mesh had an intense scar formation with considerable shrinkage and folding. In most cases, fixation sutures were still in place. All mesh demonstrated a chronic persisting foreign body reaction with formation of a fibrous capsule, even years after implantation. Overt infection increased the local inflam matory response and in most cases decreased the induction of connective tissue.

Table 1 Tissue response around polypropylene mesh removed for hernia recurrence, pain or infection

	Recurrence	Pain	Infection
Duration of incorporation (months)	29·8(30·4)	28·3(17·0)	14·2(12·8)*
Partial volume of inflammatory infiltrate (%)	34·8(12·1)	34·6(12·4)	44·9(9·5)*
Partial volume of connective tissue (%)	41·2(8·6)	35·0(8·8)	30·8(11·7)†
Partial volume of vessels (%)	9·8(2·3)	9·6(2·4)	8·3(2·3)†
Macrophages	36·6(7·9)	35·5(5·8)	44·5(11·6)*
Polymorphonuclear granulocytes	7·8(3·1)	7·4(4·8)	18·0(3·6)*
Lymphocytes	7·0(3·2)	7·0(2·5)	9·0(3·8)†
Fibroblasts	16·7(6·5)	16·6(7·4)	13·0(5·3)†
TUNEL (% positive)	24·6(12·4)	24·5(9·1)	40·6(15·6)*
Ki67	21·1(9·5)	23·2(11·2)	31·5(13·9)†
HSP 70	50·6(21·7)	45·5(21·9)	23·7(14·1)*

Values are mean(s.d.). HSP, heat-shock protein. *$P < 0{\cdot}05$ *versus* recurrence or pain; †$P < 0{\cdot}05$ *versus* recurrence (least significant difference Bonferroni test)

A predominant foreign body reaction with typical foreign body granulomas including epitheloid cells and giant cells was observed. Smaller numbers of PMNs were detected regularly at the interface between mesh and recipient tissues. The inflammatory process was accompanied by a pronounced perifilamentous fibrosis with extensive deposition of collagen fibres. In the interface of the mesh the collagen fibres were basically orientated parallel to the polypropylene threads. In addition, the pores were filled with collagen rich connective tissue. The polypropylene mesh was integrated in a complete three dimensional scar plate.

Tissue and cellular response (*Fig. 2*)

There were no significant differences between mesh removed for pain and mesh removed for recurrence with regard to inflammatory partial volume, partial volume of vessels and connective tissue, and the number of macrophages, PMNs, lymphocytes, fibroblasts and cells positive for TUNEL, Ki67 or HSP 70.

Comparison of all data indicated a significant effect of incorporation interval ($P < 0{\cdot}01$), with a decrease in the partial volume of inflammatory cells ($r = 0{\cdot}651$). In contrast, the amount of connective tissue ($r = 0{\cdot}189$) and the partial volume of vessels remained constant over time ($r = 0{\cdot}047$). The amount of fat tissue correlated inversely with the level of scar formation ($r = 0{\cdot}556$).

The time course of the inflammatory infiltrate corresponded to a similar decrease in the relative cell numbers in the interface between surgical mesh and recipient tissues, in particular of macrophages ($r = 0{\cdot}478$) and PMNs ($r = 0{\cdot}522$). Simultaneously, the local cell turnover was markedly lowered (Ki67; $r = 0{\cdot}494$). The number of TUNEL positive cells in the interface decreased with time ($r = 0{\cdot}508$, $P < 0{\cdot}01$), although in the case of mesh infection, at a raised level ($P < 0{\cdot}01$). Over time the number of fibroblasts remained almost constant or even decreased ($r = 0{\cdot}161$; $P = 0{\cdot}16$). In contrast, a marked increase in HSP 70 positive cells was detected ($r = 0{\cdot}530$) with increasing implantation interval.

A high partial volume of inflammatory infiltrate ($r = 0{\cdot}592$, $P < 0{\cdot}01$) with increased numbers of macrophages ($r = 0{\cdot}389$, $P < 0{\cdot}01$) and PMNs ($r = 0{\cdot}701$, $P < 0{\cdot}01$) was significantly associated with an increased level of TUNEL positive and proliferating cells ($r = 0{\cdot}619$, $P < 0{\cdot}01$) and, in contrast, to a decreased expression of HSP 70 ($r = 0{\cdot}812$, $P < 0{\cdot}01$) as a marker of the cell stress response.

Finally, the presence of infection led to a significant increase in macrophages, PMNs and lymphocytes and a decrease in fibroblasts ($P < 0{\cdot}05$).

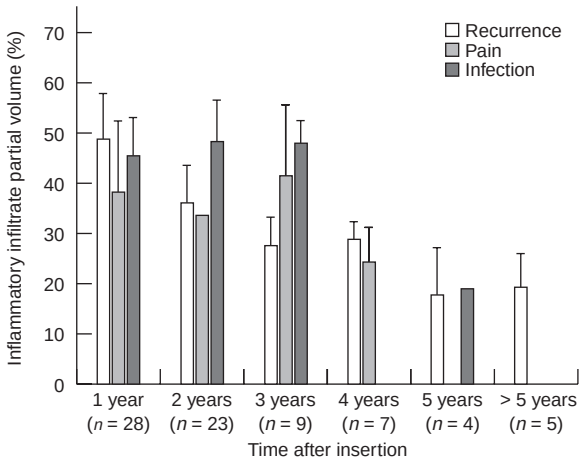
Discussion

Modern surgical hernia repair depends increasingly on synthetic mesh for reconstruction of the abdominal wall. Despite the undisputed advantage of the polypropylene mesh currently available, reports of complications after implantation are increasing. Minor local complaints such as seroma, discomfort and decreased abdominal wall mobility are frequent, and observed in about one half of patients. Serious complications such as recurrence, chronic and persisting pain and infection, including fistula formation, are rare[17–20], but sometimes force a surgeon to remove the surgical mesh. Mesh specimens obtained as a result of reoperation offer a unique insight into the long term behaviour of these implants and therefore an overview of their biocompatibility.

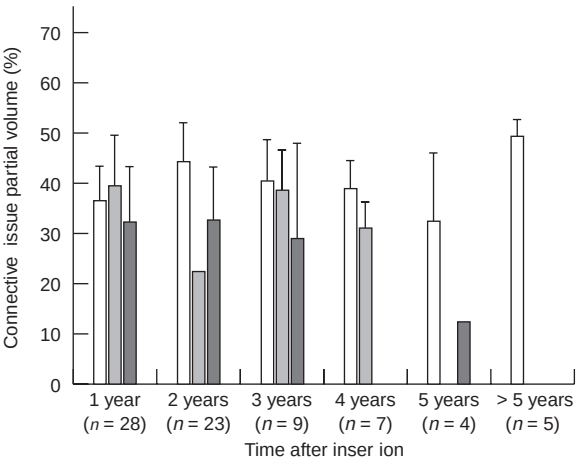
It is surprising that data about the long term biocompatibility of surgical mesh, which has now been in clinical use for more than 40 years, are not available. Mesh now represents numerically the largest group of alloplastic implants most frequently used in modern medicine. Since 1995 the authors have systematically collected and analysed explanted surgical mesh specimens. By 2001 approximately 350 samples of various mesh modifications had been gathered from hernia centres from all over Europe. In the present study a group of 76 Marlex® mesh specimens was selected to investigate the local reaction of tissues in contact with the mesh. The main questions to be answered were whether the tissue response changed over time and whether morphological data could be correlated with the reason for mesh removal. It should be stressed that the findings cannot simply be extrapolated to mesh in uncomplicated hernias; failure of mesh repair due to technical problems during the operation cannot be excluded in this study.

Analysis of the local tissue response indicated a uniform reaction of all patients to the polypropylene mesh. While the quantity and severity of the typical inflammatory reaction varied considerably between patients, the nature of the reaction was identical. Moreover, the tissue response in humans was almost identical to the morphological observations in various animals models[3,5,12,13,15,21]. These data clearly document an increase in the biocompatibility of polypropylene mesh during long term implantation. Over time, there was a decreased inflammatory reaction and an increase in HSP 70 expression running inversely to the level of inflammation[22]. Even 15 years after implantation, the longest observation in this study, a persisting chronic foreign body reaction could still be detected, implying that mesh is never completely inert with respect to local inflammatory processes.

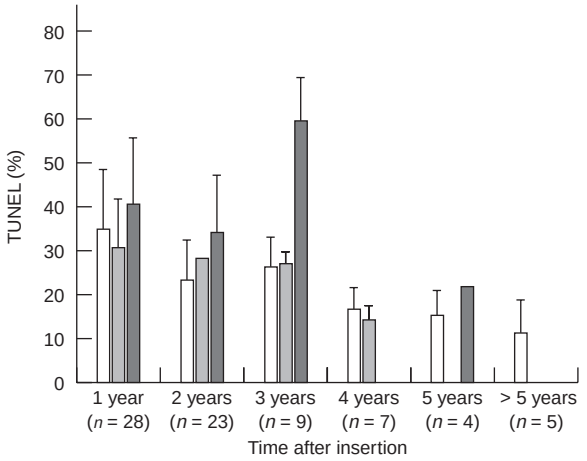
In conjunction with the increasing biocompatibility during implantation, most of the serious complications



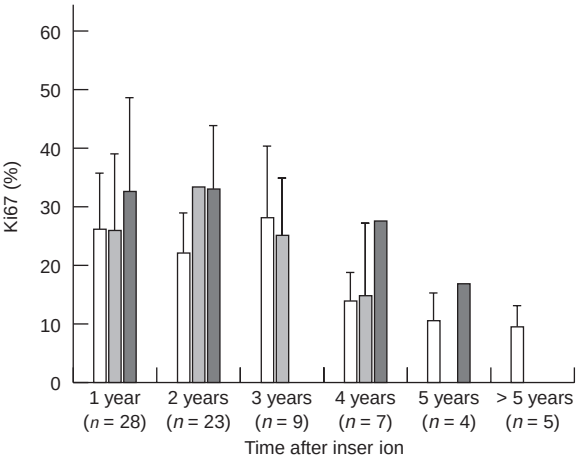
a Inflammatory cells



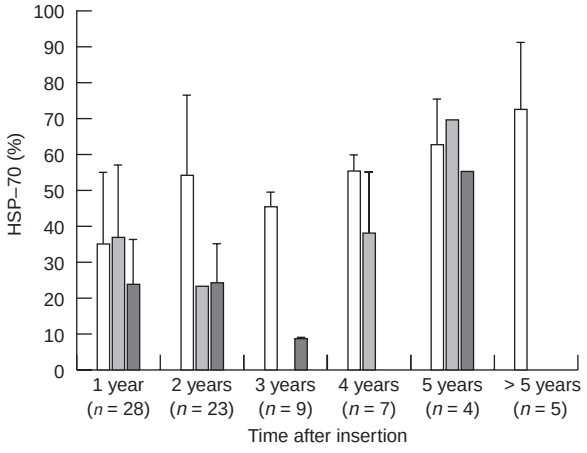
b Connective tissue



c TUNEL-positive cells



d Ki67



e Heat-shock protein

Fig. 2 Tissue and cellular response to standard polypropylene mesh removed for recurrent hernia, pain or infection according to time after insertion. Values are mean(s.d.). **a** Partial volume of all inflammatory cells, **b** partial volume of connective tissue formation, **c** percentage of TUNEL positive cells, **d** percentage of Ki67 positive cells and **e** percentage of heat shock protein (HSP) 70 positive cells

that required reoperation, namely recurrence, chronic pain or infection, occurred within 3 years of implantation. Whether the increased foreign body reaction and inflammatory response in the first 3 years of mesh incorporation is causally related to the observed rate of serious complications, or whether it just reflects the selection of mesh failures for study, remains open to conjecture. Complications 5 years after implantation were extremely rare in all mesh samples recorded in Aachen. However, the delay before explantation of mesh for infection of up to 56 months, for chronic pain of up to 48 months and for recurrence of up to 180 months indicated that in many clinical studies the morbidity rates are underestimated.

Not surprisingly, the tissue response was most affected by concomitant infection, leading to a significantly enhanced inflammatory reaction. However, it was striking that there was little difference in inflammatory response in mesh removed for recurrence or chronic pain, contradicting the possibility of a specific tissue reaction as an underlying cause for either complication.

Acknowledgements

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